

MECHANISMS OF PHAGOCYTOSIS IN MACROPHAGES

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ABSTRACT

Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive response. In order to discriminate between infectious agents and self, macrophages have evolved a restricted number of phagocytic receptors, like the mannose receptor, that recognize conserved motifs on pathogens. Pathogens are also phagocytosed by complement receptors after relatively nonspecific opsonization with complement and by Fc receptors after specific opsonization with antibodies. All these receptors induce rearrangements in the actin cytoskeleton that lead to the internalization of the particle. However, important differences in the molecular mechanisms underlying phagocytosis by different receptors are now being appreciated. These include differences in the cytoskeletal elements that mediate ingestion, differences in vacuole maturation, and differences in inflammatory responses. Infectious agents, such as *M. tuberculosis*, *Legionella pneumophila*, and *Salmonella typhimurium*, enter macrophages via heterogeneous pathways and modify vacuolar maturation in a manner that favors their survival. Macrophages also play an important role in the recognition and clearance of apoptotic cells; a notable feature of this process is the absence of an inflammatory response.

INTRODUCTION

Cells have evolved a variety of strategies to internalize particles and solutes, including pinocytosis, receptor-mediated endocytosis, and phagocytosis (reviewed in 1-4). Pinocytosis usually refers to the uptake of fluid and solutes, and it is closely related to receptor-mediated endocytosis, the specific

593

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process through which macromolecules, viruses, and small particles enter cells. Pinocytosis and receptor-mediated endocytosis share a clathrin-based mechanism and usually occur independently of actin polymerization. By contrast, phagocytosis, the uptake of large particles ($>0.5 \mu\text{m}$) into cells, occurs by an actin-dependent mechanism and is usually independent of clathrin. While lower organisms use phagocytosis primarily for the acquisition of nutrients, phagocytosis in Metazoa occurs primarily in specialized phagocytic cells such as macrophages and neutrophils, and it has evolved into an extraordinarily complex process underlying a variety of critical biological phenomena. Thus, phagocytosis by macrophages is critical for the uptake and degradation of infectious agents and senescent cells, and it participates in development, tissue remodeling, the immune response, and inflammation. Monocytes/macrophages and neutrophils have been referred to as professional phagocytes and are very efficient at internalizing particles. On the other hand, most cells have some phagocytic capacity. For example, thyroid and bladder epithelial cells phagocytose erythrocytes *in vivo*, and numerous cell types have been induced to phagocytose particles in culture. A group of cells termed paraprofessional phagocytes by Rabinovitch (who also coined the terms professional and nonprofessional phagocytes) have intermediate phagocytic ability (3). These include retinal epithelial cells that internalize the effete ends of retinal rods (3). The major difference with respect to phagocytic capacity and efficiency of professional and nonprofessional phagocytes can probably be ascribed to the presence of an array of dedicated phagocytic receptors that increase particle range and phagocytic rate. Transfection of fibroblasts and epithelial cells with cDNAs encoding Fc receptors (FcRs) dramatically increases the phagocytic rate (and, obviously, particle range) (5), and this system has been used to dissect signaling pathways leading to particle internalization. However, it is clear that many other differences between professional and nonprofessional phagocytes exist that lead to the enhancement of both rate and efficiency of particle internalization. The study of phagocytosis requires insight into the mechanisms of signal transduction, actin-based motility, membrane trafficking, and infectious disease. While a basic description of phagocytosis has been available since the seminal studies of Metchnikoff (6), investigations conducted over the last decade have begun to unravel the molecular basis of this process. In this overview, we limit our focus to phagocytic mechanisms in macrophages. Other phagocytic cells such as neutrophils certainly use similar mechanisms, but important differences exist that may be important to the role each cell type plays in the immune response.

Phagocytosis is extremely complex, and no single model can fully account for the diverse structures and outcomes associated with particle internalization. This complexity is in part due to the diversity of receptors capable of stimulating phagocytosis, and in part due to the capacity of a variety of microbes to influence

their fate as they are internalized. The fact that most particles are recognized by more than one receptor, and that these receptors are capable of cross-talk and synergy, further complicates our understanding. In addition, many phagocytic receptors have dual functions, often mediating both adhesion and particle internalization, and a complex relationship exists between these two related processes. Adhesion receptors and phagocytic receptors can both activate and inhibit each other's function. For example, ligation of the fibronectin receptor ($\alpha_5\beta_1$ integrin) at the substrate-adherent surface of a monocyte establishes preconditions within the cell that permit the otherwise inactive complement receptor CR3 ($\alpha_M\beta_2$ integrin) to mediate phagocytosis (7, 8). On the other hand, adherent cells often round up during phagocytosis, implying that there is competition for cytoskeletal and membrane components necessary for phagocytosis and adhesion. This notion is reinforced by the observation that many of the cytoskeletal components known to participate in adhesion are also enriched in the phagocytic cup. These include paxillin, talin, vinculin, α -actinin, protein kinase C α , MARCKS and MacMARCKS (9, 10).

Despite the complexity associated with different phagocytic mechanisms, a number of shared features follow: Particle internalization is initiated by the interaction of specific receptors on the surface of the phagocyte with ligands on the surface of the particle. This leads to the polymerization of actin at the site of ingestion, and the internalization of the particle via an actin-based mechanism. After internalization actin is shed from the phagosome, and the phagosome matures by a series of fusion and fission events with components of the endocytic pathway, culminating in the formation of the mature phagolysosome. Since endosome-lysosome trafficking occurs primarily in association with microtubules, phagosome maturation requires the coordinated interaction of the actin and tubulin based cytoskeletons.

RECEPTORS: MECHANISMS OF RECOGNITION

A primary challenge to the innate immune system is the discrimination of a large number of potential pathogens from self, utilizing a restricted number of phagocytic receptors. This problem is compounded by the propensity of pathogens to mutate. This challenge has been met by the evolution of a variety of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes. These motifs have essential roles in the biology of the invading agents, and they are therefore not subject to high mutation rates. Janeway has proposed calling the receptors "pattern-recognition receptors" (PRRs) and the targets for these receptors "pathogen-associated molecular patterns" (PAMPs) (11). Pathogen-associated motifs include mannans in the yeast cell wall, formylated peptides in bacteria, and lipopolysaccharides and

lipoteichoic acids on the surface of Gram negative and Gram positive bacteria. The recognition mechanisms leading to phagocytosis occur either cellularly or humorally. Cellular receptors that recognize these patterns include the mannose receptor (MR) and DEC 205 that recognize mannans, as well as integrins (for example CD11b/CD18) and scavenger receptors that recognize surface components on bacteria including LPS (12, 13). Humoral components that opsonize the infectious agent before being recognized by a phagocytic receptor include the mannose-binding protein, which binds mannans and is recognized by the C1q receptor, and surfactant protein A, which binds carbohydrates and is recognized by a transmembrane receptor, SPR210 (14, 15). Antibodies represent an intersection between adaptive and innate immunity: They recognize their cognate ligands on infectious agents with exquisite specificity but are bound and internalized through their generic Fc domains by the Fc family of receptors (FcRs) (16–18). The complement system lies somewhere in between: The C3bi receptor binds to the C3bi fragment that is fixed nonspecifically to the carbohydrate surface of pathogens via the alternative pathway (19, 20). Alternatively, complement is fixed to IgM that specifically recognizes epitopes on the surface of the pathogen.

Fc Receptor-Mediated Phagocytosis

Most of our understanding of the signaling pathways leading to phagocytosis in macrophages comes from studies of the FcR (16–18). FcRs fall into two general classes—those involved in effector functions and those that transport immunoglobulins across epithelial barriers. There are two major classes of Fc γ receptors: receptors that activate effector functions and receptors that inhibit these functions (16, 17, 21). FcRs that mediate phagocytosis in human macrophages fall within the activation class and include Fc γ RI, Fc γ RIIA, and Fc γ RIII (Figure 1) (17). The human Fc γ RIIA is a single chain protein with an extracellular Fc binding domain, a transmembrane domain, and a cytoplasmic tail containing two YXXL ITAM motifs (for immunoglobulin gene family tyrosine activation motif) similar to those found in T cell and B cell receptors (22, 23). There is no mouse counterpart to Fc γ RIIA. Murine macrophages, as well as human macrophages, express Fc γ RIIB, an inhibitory receptor that does not contain ITAM motifs and does not participate in phagocytosis (22, 24). Ligand binding results in receptor cross-linking, and this causes tyrosine phosphorylation of the ITAMs (see below). Fc γ RI and Fc γ RIIIA have extracellular Fc binding domains similar to the Fc γ RIIA, but lack ITAMs on their cytoplasmic tails (22). For proper expression and signaling, these receptors must interact with a dimer of γ subunits (Fc γ RI and Fc γ RIIIA), or ζ subunits (Fc γ RIIIA), small transmembrane proteins that contain the ITAMs needed for signal transduction (Figure 1) (25, 26). Ligation of Fc γ receptors I or III

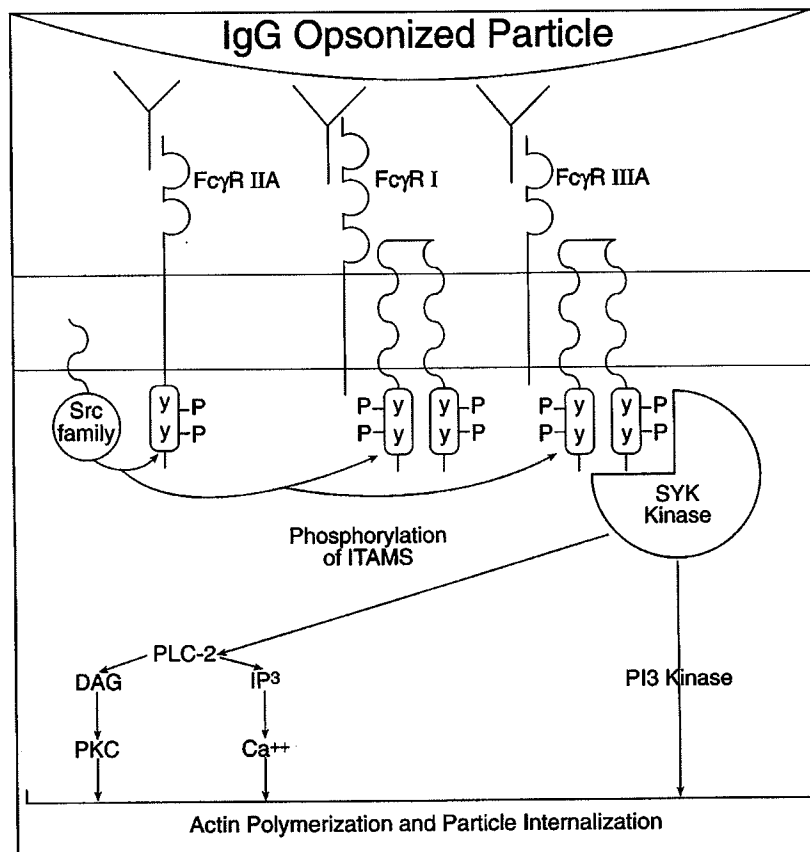


Figure 1 Fc γ receptors signal phagocytosis via their phosphorylated ITAM domains. Receptor cross-linking stimulates src family kinases to phosphorylate tyrosine (Y) residues within the ITAM domain of Fc γ R IIA or within the dimerized γ subunits of Fc γ R I or Fc γ R IIIA. The tyrosine kinase syk is then recruited to the phosphorylated ITAM domain, and upon its activation, it is thought to mediate particle internalization by activating PI3-kinase and phospholipase C.

results in their cross-linking and in the tyrosine phosphorylation of the ITAM domains of their γ subunits (22). Deletion of the gene encoding the γ subunit of Fc γ receptor in mice results in macrophages that are unable to express Fc γ R I or III, since these receptors are not transported to the surface of cells in the absence of their signaling subunit, and macrophages from these mice are unable to phagocytize IgG-coated particles (26).

The role of the ITAM motifs of the γ subunit of the Fc γ receptor have also been analyzed in COS cells, where all three members of the Fc γ R family

are capable of promoting phagocytosis (5). Since COS cells are not professional phagocytes, and all cells have some capacity to phagocytose (as discussed above), it is likely that much more than the presence or absence of FcRs is responsible for efficient phagocytosis by professional phagocytes. Indeed, COS cells expressing FcRs phagocytose IgG-opsonized particles much less efficiently than macrophages. However, many of the early signaling events may be reconstituted in COS cells. In transfected COS-1 cells, Fc γ RIIIA or I mediates phagocytosis of IgG-opsonized particles, but only when coexpressed with the γ chain, and the ITAM motif of γ is required for a competent phagocytic signal (5). The ζ chain of the T cell receptor contains sequences homologous to the γ chain, including the conserved YXXL motifs, and can substitute for the γ chain in Fc γ RIIIA-dependent signaling of phagocytosis (27). However, the ζ chain is considerably less efficient in mediating Fc γ RIIIA-dependent phagocytosis than is the γ chain, and mutational analysis demonstrates that the functional differences between the γ and ζ subunits are due to the internal amino acids of the YXXL (27, 5). Cross-linking of Fc γ RIIIA results in tyrosine phosphorylation of the γ subunit, and mutation of either tyrosine of the two YXXL motifs of the γ subunit ITAM eliminates both tyrosine phosphorylation and phagocytosis (5, 27, 28).

The protein tyrosine kinase responsible for this initial phosphorylation is thought to be a member of the src family (29, 30). Subsequently, a second protein tyrosine kinase, p72Syk, is recruited to the phosphorylated ITAM domains (22, 30–33). This results in the activation of the Syk kinase, which in turn triggers a plethora of pathways leading to transcriptional activation, cytoskeletal rearrangement, and the release of inflammatory mediators.

This model is supported by the observation that a chimera containing the extracellular domain of CD16 (Fc γ RIII), fused to the transmembrane stalk of CD7 and containing p72syk intracellularly, is capable of signaling phagocytosis of IgG opsonized particles in transfected COS cells (34). A competent phagocytic stimulus was independent of Syk SH2 domains but required an active Syk kinase (34). The related tyrosine kinase ZAP70 could substitute for Syk in this system, whereas members of the src family of tyrosine kinases could not (34). These studies have been extended in DT40 lymphocytes, a chicken cell line that has been valuable in dissecting signaling pathways because it undergoes a high rate of homologous recombination and therefore permits gene deletions at high frequency. DT40 cells, expressing a fusion protein consisting of the extracellular domain of human Fc γ RIIIA and the ITAM-containing γ subunit of the Fc receptor, are capable of localized actin polymerization when the chimeric receptors are clustered (35). Actin assembly is dependent upon an intact ITAM, absent in cells lacking Syk and exacerbated in cells overexpressing Syk (35), suggesting an absolute requirement for the Syk tyrosine kinase

in ITAM-dependent actin assembly in DT40 cells. The requirement for Syk can probably be extended to all hematopoietic cells, since FcR-mediated actin assembly and phagocytosis is abrogated in macrophages derived from the fetal livers of Syk null mice (36). Further evidence for the involvement of Syk in phagocytosis is also derived from the COS cell system. Upon cross-linking of Fc γ R1IIIA/ γ and Fc γ R1/ γ , Syk is phosphorylated and enhances the phagocytosis of IgG-opsonized erythrocytes; this activity is dependent on the γ chain (37, 5). Both SH2 domains of Syk are necessary for functional association with the γ subunit, and Syk is unable to induce either Fc γ R1 or Fc γ R1IIIA mediated phagocytosis by γ chain mutants in which YXXL tyrosine is replaced by phenylalanine (5, 37). How the Syk tyrosine kinase stimulates actin assembly is unknown, although it is likely that PI 3-kinase is involved (see below).

There are clearly a number of problems with this model. First, it only applies to Fc γ R-mediated phagocytosis. Thus, while macrophages from syk-mice are incapable of Fc γ R-mediated phagocytosis, phagocytosis of latex particles, yeast, and *E. coli* is unimpaired (36). Second, macrophages derived from mice deficient in the three members of the Src-family kinases known to be expressed in these cells, Hck, Fgr, and Lyn, exhibit poor Syk activation when the Fc γ R is ligated but are still capable of Fc γ R-mediated phagocytosis, albeit at a slightly slower rate (36). Either a small activation of Syk is sufficient to support Fc γ R-mediated phagocytosis, or Syk participates by another means, perhaps by serving as an adapter. An answer to this conundrum might be found in the interesting observation that the c-fgr tyrosine kinase actually suppresses phagocytosis in macrophages (H Gresham, C Willman, unpublished data). These investigators found that while a fgr-negative murine macrophage line phagocytosed normally, Fc γ R1, Fc γ R1I/Fc γ R1II, and C3bi-mediated phagocytosis was suppressed when the cells were transfected with wild-type c-fgr. While actin rearrangement and phagocytosis is suppressed, c-fgr has no effect on receptor expression or on attachment of the opsonized particle. The suppressive effect of c-fgr is independent of its kinase activity, implying that inhibition of phagocytosis may be mediated through an adapter function.

DOWNSTREAM EFFECTORS OF FC RECEPTOR-MEDIATED PHAGOCYTOSIS The mechanism by which Fc γ Rs stimulate the polymerization of actin and induce the formation of phagosomes is not known, although PI-3 kinase, the rho family of GTPases, protein kinase C (PKC), and motor proteins appear to participate.

PI-3 kinase Recent evidence suggests that PI 3-kinase participates in the signaling cascade of phagocytic receptors. PI 3-kinase catalyzes phosphorylation at the D-3 position of the inositol ring of phosphatidylinositol (PI), PI(4)P and PI(4,5)P₂, and is activated by many tyrosine kinase receptors that trigger the

polymerization of actin (38). In addition, there is compelling evidence in yeast that PI 3-kinase participates in membrane trafficking (39). Cross-linking of Fc γ RI and RII increases PI 3-kinase activity, and FcR-mediated phagocytosis is prevented by wortmannin or LY294002, specific inhibitors of PI 3-kinase (40, 41). In addition, stimulation of Fc γ RIIA in platelets causes the association of the receptor with PI 3-kinase (42). Elegant studies by Swanson and colleagues indicate that wortmannin and LY294002 PI do not inhibit actin-dependent formation of the phagocytic cup, but instead prevent the phagosome from sealing behind the particle (41). De Franco and coworkers confirmed these data and further demonstrated that macrophages from syk null mice are similarly capable of polymerizing actin beneath the Fc γ R-induced phagocytic cup but are unable to complete internalization (36). This suggests that PI 3-kinase may participate in a syk-dependent signaling pathway critical for Fc γ R-mediated phagocytosis.

GTPases Members of the Rho family of GTPases have been shown to regulate the actin cytoskeleton in response to a variety of extracellular signals (43). In 3T3 cells, various members of the Rho family act hierarchically during cell spreading: Cdc42 participates in the formation of filopodia and in the activation of Rac; Rac stimulates membrane ruffling and activates Rho, and Rho stimulates the formation of focal adhesions and stress fibers (44). Recent evidence demonstrates that the Rho family also participates in phagocytosis. Microinjection of the J774 mouse macrophage cell line with the Rho-specific inhibitor C3 exotoxin inhibits Fc γ R-mediated phagocytosis by preventing receptor clustering, a prerequisite for efficient particle binding and internalization (45). By contrast, inhibition of Rac1 and Cdc42, by expression of their dominant negative forms in the RAW mouse macrophage cell line, does not affect particle binding to FcRs, but inhibits phagocytosis by preventing the accumulation of F-actin in the phagocytic cup (46). The precise mechanism by which these GTPases regulate F-actin structure has not yet been defined, but a variety of cytoskeletal regulators including PIP 5-kinase and myosin II have been implicated (43). Members of the ARF family of GTPases have a role in most membrane trafficking events. ARF6 has been implicated in endocytosis, membrane recycling and regulated exocytosis (47). Expression of a mutant form of ARF6 that is incapable of hydrolyzing GTP causes profound rearrangement of F-actin in HeLa cells (48), and inhibits Fc γ R-mediated phagocytosis in a macrophage cell line (49).

Protein kinase Cts Protein kinase C (PKC) also appears to have a role in phagocytosis (10, 50, 51), previous studies demonstrated that PKC is activated upon ligation of the Fc γ R in human monocytes (50) and localizes the α isozyme of PKC to nascent phagosomes in macrophages (10). The involvement of PKC

in phagocytosis is tantalizing since its major substrate, MARCKS, is known to regulate actin structure at the membrane (52). MARCKS is rapidly phosphorylated during particle uptake, and MARCKS and PKC α are recruited to the forming zymosan phagosome with kinetics similar to those of F-actin (10). MARCKS cross-links F-actin, and this activity is prevented by PKC-dependent phosphorylation and by calcium/calmodulin (53). Since the association of MARCKS with membranes is also regulated by PKC-dependent phosphorylation, it is an ideal candidate to regulate actin structure on the phagosome in response to signals from both PKC and calcium/calmodulin. This is supported by the observation that inhibitors of PKC prevent phagocytosis and block the accumulation of PKC α , MARCKS, F-actin, and a number of other cytoskeletal proteins beneath bound zymosan (10).

MacMARCKS, another member of the MARCKS family, also associates with zymosan phagosomes (54, 55), and a mutant form of MacMARCKS appears to block phagocytosis when expressed in a macrophage cell line (55). The significance of this observation is unclear since macrophages derived from MacMARCKS null mice phagocytose zymosan normally (56).

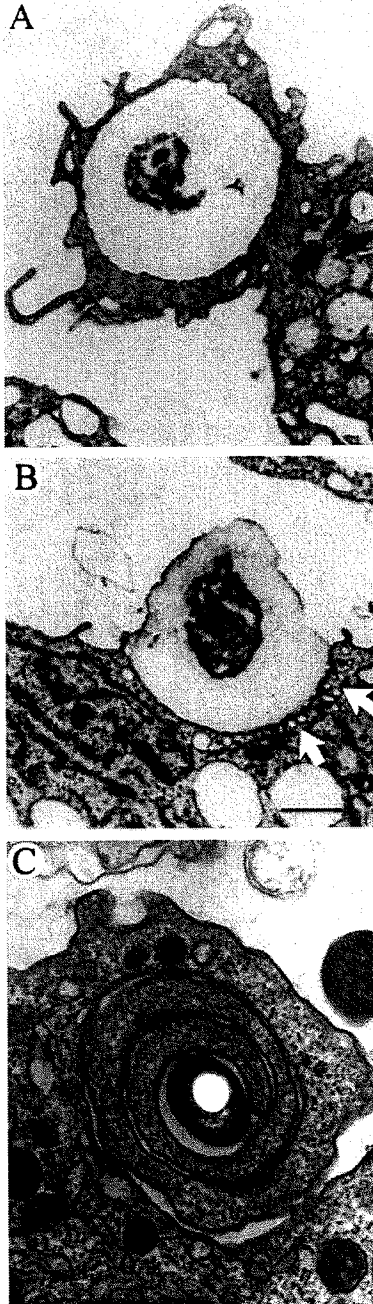
Motor proteins It is not clear whether actin polymerization alone is sufficient to drive pseudopod extension and particle internalization, or whether this also requires molecular motors. It has long been known that myosin II accumulates on the phagocytic cups of macrophages and neutrophils ingesting yeast, implying that it might act as a mechanical motor during particle internalization (57). Myosin I, myosin V, and myosin IX also colocalize with F-actin on forming phagosomes, suggesting that it too might facilitate ingestion (10, 58). Despite these colocalization studies and the observation that the broad spectrum myosin inhibitor BDM prevents phagocytosis (58) (D Underhill, unpublished observations), there is as yet no information on the specific roles of myosin isoforms in phagocytosis.

Complement Receptor-Mediated Phagocytosis

Complement proteins, present in serum, opsonize bacteria for phagocytosis by the C3b or C3bi receptors (CRs) on macrophages. Several receptors that participate in phagocytosis of complement-opsonized particles, including CR1, CR3, and CR4 are expressed on macrophages (19, 20). CR1 is a single chain transmembrane protein consisting of a large extracellular lectin-like complement-binding domain and a short 43 amino acid cytosolic domain. CR1 binds C3b, C4b, and C3bi, and is thought to participate mainly in particle binding (59). CR3 and CR4 are integrin family members made up of heterodimers of different α chains (α_m for CR3 and α_x for CR4) and a shared β chain (β_2) (19). These two receptors bind specifically to C3bi and are responsible for particle internalization.

While FcRs are constitutively active for phagocytosis (22), the CRs of resident peritoneal macrophages bind but do not internalize particles in the absence of additional stimuli (7, 8). Particle ingestion by CRs can be induced by PKC activators such as PMA, as well as by TNF- α , granulocyte/macrophage colony-stimulating factor (GM-CSF), or attachment to laminin- or fibronectin-coated substrata (7, 8, 60). Although all types of phagocytosis require actin polymerization at the site of ingestion (2), results of electron microscopy (EM) studies demonstrate that IgG- and complement-opsonized particles are internalized differently by macrophages (9, 61). During Fc γ R-mediated phagocytosis, veils of membrane rise above the cell surface and tightly surround the particle before drawing it into the body of the macrophage (9, 61) (Figure 2A). Silverstein and colleagues have demonstrated that Fc γ R-mediated ingestion occurs by a zipper process, in which Fc γ Rs in the macrophage plasma membrane interact sequentially with IgG molecules distributed over the surface of the ingested particle (1). On the other hand, EM data indicate that CR-mediated phagocytosis is a relatively passive process that occurs by a variation of the classic zipper model; complement-opsonized particles appear to sink into the cell with elaboration of small, if any, pseudopodia (9, 61) (Figure 2B). Moreover, the phagosome membrane is less tightly opposed to complement-opsonized particles, with point-like contact areas separating regions of looser membrane. These point-like contact areas are enriched with a variety of cytoskeletal proteins including F-actin, vinculin, α -actinin, paxillin, and phosphotyrosine-containing proteins, and their formation is blocked by inhibitors of PKC, but not by inhibitors of protein tyrosine kinases (although tyrosine phosphorylation increases the efficiency of phagocytosis) (9) (Figure 3E and F, and data not shown). By contrast, all of these proteins are diffusely distributed on phagosomes containing IgG-coated particles (Figure 3C and D), and Fc γ R-mediated phagocytosis is blocked by both PKC and tyrosine kinase inhibitors (9). Thus, the signals required for particle ingestion and the arrangement of cytoskeletal proteins on the phagosome surface vary depending upon which phagocytic receptor is engaged. Moreover, complement receptor (CR)-mediated internalization requires intact

Figure 2 Different particles are internalized by distinct phagocytic mechanisms. Cryo-EM sections of peritoneal macrophages that are in the process of ingesting IgG-opsonized particles (A) or complement-opsonized particles (B). Note that pseudopodia protrude from the macrophage surface to engulf the IgG-opsonized particle, whereas the complement-coated particle sinks directly into the cell. Arrows in B indicate vesicles directly beneath the forming complement phagosome that are absent beneath the FcR phagosome in A. [Reprinted with permission from *The Journal of Experimental Medicine* (LAH Allen, A Aderem. 1996. *J. Exp. Med.* 184:627-37).] (C) *L. pneumophila* is internalized into human monocytes by coiling phagocytosis. [Reprinted with permission from *Cell* (MA Horwitz. 1984. *Cell* 36:27-33).]



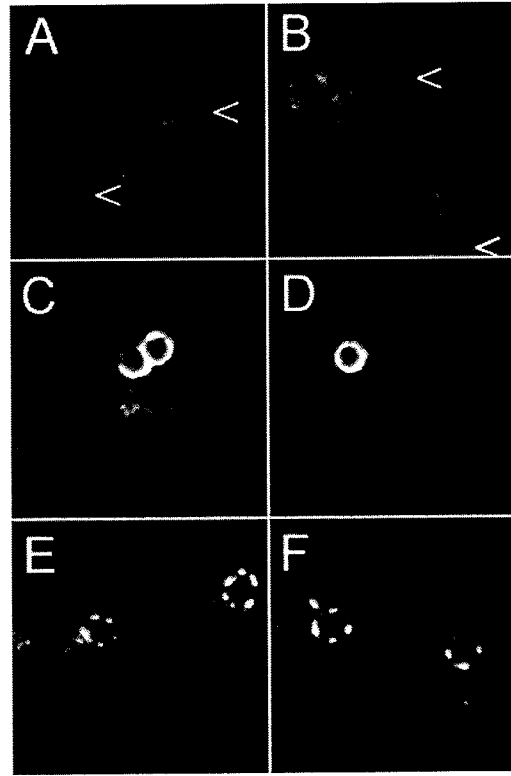


Figure 3 Vinculin and paxillin associate differently with different types of phagosomes. Zymosan-containing phagosomes (*A* and *B*) are not stained with antibodies to vinculin (*A*) or paxillin (*B*). The arrowheads indicate the position of the phagosomes. FcR-mediated phagosomes (*C* and *D*) are enriched with vinculin (*C*) and paxillin (*D*) in a uniform pattern, while complement receptor-mediated phagosomes (*E* and *F*) are coated with vinculin (*E*) and paxillin (*F*) in discrete foci. [Reprinted with permission from *The Journal of Experimental Medicine* (LAH Allen, A Aderem. 1996. *J. Exp. Med.* 184:627–37).]

microtubules and is accompanied by the accumulation of vesicles beneath the forming phagosome (Figure 2*B*, arrows), suggesting that membrane trafficking plays a key role in CR-mediated phagocytosis (9).

An additional difference between FcR- and CR-mediated phagocytosis relates to their capacity to trigger the release of inflammatory mediators. FcR-induced phagocytosis is tightly coupled to the production and secretion of proinflammatory molecules such as reactive oxygen intermediates and arachidonic acid metabolites (62, 63). By contrast, CR-mediated phagocytosis does not elicit the release of either of these classes of inflammatory mediators (62, 63).

Mannose Receptor-Mediated Phagocytosis

The mannose receptor (MR) on macrophages recognizes mannose and fucose on the surfaces of pathogens and mediates phagocytosis of the organisms (13). The high affinity of this receptor for branched mannose and fucose oligosaccharides, prototypic PAMPS as described above, makes the MR a phagocytic receptor with broad pathogen specificity.

The MR is a single chain receptor with a short cytoplasmic tail and an extracellular domain including 8 lectin-like carbohydrate-binding domains. The lectin-like carbohydrate-binding domains share homology with other C-type lectins including the mannose-binding protein, collectins, DEC 205, and the phospholipase A2 receptor (64, 65). The cytoplasmic tail is crucial to both the endocytic and phagocytic functions of the receptor, but little is known about the signals that lead to phagocytosis (13, 64).

During mannose receptor-mediated phagocytosis of zymosan, the actin cytoskeleton is mobilized around the nascent phagosomes, and proteins such as F-actin, talin, PKC α , MARCKS, and Myosin I are recruited (9). However, in contrast to FcR- and CR-mediated phagocytosis, vinculin and paxillin are not recruited to MR phagosomes (Figure 3*A* and *B*), reinforcing the notion that different phagocytic receptors send different signals to the actin cytoskeleton and initiate different mechanisms of internalization (9).

In addition to the phagocytic signals mediating particle internalization, pro-inflammatory signals are generated upon MR ligation. Specifically, IL-1 β , IL-6, GM-CSF (66), TNF- α (67, 68), and IL-12 (69) are all produced. Therefore, like phagocytosis mediated by the FcR, but not the complement receptor, MR-mediated phagocytosis is a pro-inflammatory process. However, at least one report suggests that release of additional proinflammatory cytokines including MIP-1 β and MIP-2 is not mediated by MR signaling (66).

MATURATION OF THE PHAGOSOMAL VACUOLE

Soon after internalization, F-actin is depolymerized from the phagosome, and the newly denuded vacuole membrane becomes accessible to early endosomes (4). Through a series of fusion and fission events, the vacuolar membrane and its contents mature, fusing with late endosomes and ultimately lysosomes, to form a phagolysosome.

The rates of phagosome-lysosome fusion vary dramatically depending on the nature of the ingested particle; during FcR- and MR-mediated uptake the phagosome fuses with lysosomes within 30 min, while phagosomes containing latex particles might not fuse with lysosomes for hours (70–74). The rate at which phagosomes mature may be related to the nature of the interaction between the particle surface and the phagosomal membrane; for example, phagosomal

membranes are in close apposition to *Mycobacteria*, and those phagosomes do not fuse with lysosomes (72). It has been proposed that the nature of the particle surface modifies the rate of vacuole maturation and that the hydrophobic surface of a *Mycobacterium* inhibits recycling or maturation of phagosomal membranes, an event required for the fusion of phagosomes and membranes (72). An important and poorly understood aspect of phagosome maturation relates to the mechanism by which the luminal contents of the phagosome are sorted. When lysosomes are preloaded with different sizes of fluorescent probes, the smaller probes report phagosome-lysosome fusion earlier than do the larger molecules (75). This size selective transfer was further investigated and led to the suggestion that transfer occurred via narrow, aqueous bridges that only permit limited content exchange (76, 77). Desjardins has suggested that transient and incomplete fusion between compartments may preserve the specific identity of each organelle, a mechanism he has called "kiss-and-run" (78). Formation of the phagolysosome is not a terminal event, and the structure has the capacity to continue fusing with lysosomes (73). Once again, the surface of the particle plays a critical role in deciding the fate of the phagosome: For example, while many IgG-coated particles remained accessible to exogenously added probes, polystyrene beads are removed from the circulation (73).

Proteins that Regulate Vacuole Maturation

The annexins are a family of proteins that bind phospholipids in a calcium-dependent manner and appear to function in membrane traffic and membrane-cytoskeleton interactions. While annexins I, II, III, and V associate with phagosomes all the time, annexin IV preferentially binds more mature vacuoles (79). The significance of the annexins in phagocytosis is not known.

The small molecular mass GTPases rab5, rab7, and rap1 sequentially associate with phagosomes as they mature (74, 80). Since homotypic fusion between early endosomes is dependent on the presence of rab5 on both organelles (81), it is likely that this mechanism also facilitates the fusion of newly formed phagosomes displaying rab5 with early endosomes. This principle can be extended. Since rab7 plays a role similar to that of rab5 further down the endocytic pathway (82), it is likely that the loss of rab5 and acquisition of rab7 by the maturing phagosome permits the fusion of the organelle with rab7-enriched late endosomes. Studies with pathogens that subvert vacuole maturation support a role for rab proteins in the process. A role for rab5 in phagosome-endosome fusion has been implicated in studies using a hemolysin-deficient strain of *Listeria monocytogenes*. Within 30 min after internalization by a macrophage, *Listeria* normally lyses the phagosomal membrane and replicates in the cytosol (83). In an in vitro fusion assay, immunodepletion of rab5 blocked phagosome-

endosome fusion, and rab5 was found specifically to accumulate on *Listeria*-containing vacuoles (84).

A number of studies have demonstrated the presence on phagosomes of a variety of proteins associated with the SNARE mechanism of membrane fusion (85), including synaptobrevins and syntaxins, but although these proteins undoubtedly play a role in phagosome-endosome fusion, the data seem to suggest that the recognition mechanisms underlying phagosome maturation are directed by the rab proteins (77, 86).

As the phagosome matures, both its membrane composition and contents are modified by its interaction with the endocytic compartment. This is well illustrated by the distribution of acid hydrolases in the cell; early endosomes contain the bulk of cathepsin H, and this enzyme is therefore acquired more rapidly by the early phagosome than are the other lysosomal enzymes (87). By contrast, late endosomes contained the bulk of cathepsin S, and this enzyme is therefore acquired later during phagosome maturation. As phagosomes mature they move into the cell on microtubules, and this trafficking gives them the opportunity to interact with various components of the endosomal system (88). Griffiths and colleagues (88) have reconstituted bidirectional phagosome movement along microtubules in vitro and have defined a number of properties of the system. Early phagosomes move more slowly than their more mature counterparts, and most phagosomes are minus-end directed. Immunodepletion studies clearly demonstrated a role for dynein and dynactin in minus-end movement of phagosomes, while plus-end movement was mediated by kinesin and its membrane receptor kinectin (88). A similar role for kinesin has been demonstrated in the radial extension of tubular lysosomes in macrophages (89).

PHAGOCYTOSIS OF PATHOGENS

Even though one of the major functions of phagocytosis is to mediate the ingestion and sterilization of infectious agents, many pathogens such as *Salmonella typhimurium*, *Legionella pneumophila*, and *Mycobacterium tuberculosis* have evolved mechanisms for survival and even growth inside macrophage vacuoles. Once again, phagocytosis of bacteria involves a large variety of heterogeneous mechanisms.

Salmonella Typhimurium Internalization Occurs by Macropinocytosis

Salmonella typhimurium is a facultative intracellular pathogen that is capable of replicating in intracellular compartments in macrophages (90). The macrophage receptors that bind *S. typhimurium* are not known, although internalization

appears to be associated with an actin-dependent mechanism. After binding to the surface of a macrophage, virulent *S. typhimurium* induces generalized membrane ruffling that results in the internalization of the bacterium into a compartment resembling a macropinosome (91). This nascent vacuole is enormous relative to the size of the bacterium and has been called a "spacious phagosome" (91). Nonvirulent mutant strains of *S. typhimurium* bind to the surface of macrophages and do not induce membrane ruffling or macropinocytosis (92); instead, these bacteria are phagocytosed into a vacuole with a tightly opposed membrane. The signaling mechanisms leading to membrane ruffling and the formation of the spacious phagosome in macrophages are unknown, but recent data demonstrate that upon binding to epithelial cells, *S. typhimurium* utilize a type III secretion system to inject the host cell with a protein, SopE, that has GDP/GTP exchange activity specific for Rac and Cdc42 (93). SopE is required for efficient entry into epithelial cells (94), but it is not known if SopE-mediated activation of Rho family proteins is required for entry into macrophages.

Once internalized, the spacious vacuole containing virulent bacteria persists, and the bacteria multiply (91). There are conflicting reports as to the maturation of the spacious vacuole; while some suggest that the spacious phagosome acquires lysosomal markers such as LAMP-1 and cathepsin L, others suggest that the spacious phagosome remains immature and does not fuse with lysosomes (95, 96). Since one group used bone marrow-derived macrophages, while the other used a macrophage cell line, the difference in vacuole fate may be attributable to differences in cell type.

Legionella pneumophila Is Internalized by Coiling Phagocytosis

L. pneumophila is a facultative intracellular pathogen that invades and replicates in macrophages (97, 98). A bacterial surface protein, MOMP (major outer-membrane protein), fixes complement component C3 to the surface of the parasite, thereby facilitating binding to the macrophage surface through complement receptors (99). Removing complement from the media, or blocking CR3, with a specific antibody prevents bacterial adhesion to the macrophage surface (99). After binding, the parasite induces the formation of an extended host cell pseudopod that spirals around the bacterium forming a structure termed a "coiling phagosome" (Figure 2C) (100). Although CR3 is enriched in coiling phagosomes, there is no evidence that these receptors signal coiling phagocytosis. Since the structure of this phagosome is very different than that induced by CR3 (discussed above), it is likely that an additional, as yet uncharacterized, *L. pneumophila* signals must be required.

Once internalized, the outer portions of the macrophage membrane coil disintegrate, leaving the bacterium in a phagosome with a single, tightly opposed

membrane (100, 101). This phagosome has unique properties; the vacuole does not acidify below pH 6.1 and does not fuse with endosomes or lysosomes (102, 103). Instead, the phagosome initially fuses with smooth vesicles, ultimately maturing into a ribosome-studded vacuole composed of endoplasmic reticulum-derived membranes (104, 105). Finally, the bacterium divides within this vacuole and ruptures the host.

The Mycobacterium tuberculosis Vacuole

A plethora of macrophage receptors have been implicated in binding and internalization of *M. tuberculosis* (106). As with other bacteria, complement fixes to the surface of the organism through the alternate pathway, allowing deposition of complement proteins C3b and C3bi, which are recognized by CR1 and CR3 (107). In the absence of factors required for activation of the alternate pathway, a surface component of *M. tuberculosis* resembling complement component C4b can bind directly to C2b and form a C3 convertase analogous to the one formed in the classical complement cascade (108). This C3 convertase catalyzes the deposition of C3b onto the surface of the organism and facilitates binding to CR1. Blocking CR drastically reduces binding and invasion of *M. tuberculosis* but does not abolish it, suggesting that other receptors participate in their uptake. Consistent with this, *M. tuberculosis* has also been demonstrated to bind to the mannose receptor and the scavenger receptor (109). In addition, surfactant protein A enhances macrophage binding and uptake of *M. tuberculosis*, probably by the surfactant protein A receptors (110).

Phagocytosis of either Erdman or H37Ra *M. tuberculosis* in the presence of autologous nonimmune serum is associated with an increase in phospholipase D activity in human monocyte-derived macrophages, and inhibition of phospholipase D prevents the uptake of the bacterium (111). *M. tuberculosis* uptake is also associated with the tyrosine phosphorylation of multiple macrophage proteins, and tyrosine kinase inhibitors suppress the phagocytosis of the bacterium (111).

Once internalized, *M. tuberculosis* resides in a membrane-bound vacuole that resists lysosomal fusion (112) and is only mildly acidified (113). Phagosomes containing *M. avium* also fail to acidify below pH 6.5, and this appears to be due to the specific exclusion of the vesicular proton-ATPase (113). The mycobacterial vacuole is not completely sequestered, however, and it is capable of interacting selectively with early endosomes (114, 115). Transferrin cycles in and out of the mycobacterial vacuole, demonstrating that the phagosome is part of a dynamic system (116, 117). Macrophages activated with interferon- γ and bacterial lipopolysaccharide (LPS) prior to ingestion of mycobacteria are able to acidify the phagosomes containing the bacteria to pH 5.3 (118). Analysis of these vacuoles demonstrate that they accumulate the proton-ATPase and

are no longer accessible to transferrin, suggesting a substantially more mature phagosome. Electron microscopy demonstrates that macrophage activation is accompanied by a coalescence of vacuoles containing single bacteria, into large vacuoles containing many mycobacteria, and this is accompanied by a substantial decrease in bacterial viability (118). Kinetic measurements indicate that acidification of the vacuole precedes the drop in microbial viability.

PHAGOCYTOSIS OF APOPTOTIC CELLS

Apoptosis, or programmed cell death, is a process crucial in the development and homeostasis of all multicellular organisms (119). Even though an enormous number of cells are continuously undergoing apoptosis in tissues of higher organisms, these dying cells are rarely observed *in vivo* due to their efficient engulfment and degradation by phagocytic cells such as macrophages (119). Since phagocytosis of apoptotic cells is a normal, ongoing process, the mechanisms enabling macrophages to recognize, bind, internalize, and degrade apoptotic cells need to function without activating the proinflammatory responses of the macrophage, as happens during phagocytosis through many of the other phagocytic receptors (119–121). Indeed, human macrophages ingesting apoptotic neutrophils fail to secrete the chemoattractants IL-8 and MCP-1 normally associated with macrophage activation, although they do secrete transforming growth factor β 1, prostaglandin E_2 and platelet-activating factor, compounds that dampen inflammatory responses (122). On the other hand, mouse peritoneal macrophages ingesting apoptotic T cells have been observed to secrete the proinflammatory chemokine MIP-2, suggesting that some degree of macrophage activation may occur under certain circumstances (123).

In order to phagocytose an apoptotic cell, receptors on the macrophage must see a ligand found on apoptotic cells that is not present on healthy cells. Ligands fitting these criteria that have been implicated in the recognition of apoptotic cells include phosphatidylserine in the outer leaflet of the plasma membrane, changes in the pattern of glycosylation of cell surface proteins, and surface charge (119, 121). Recent studies make it clear that there are many receptors (both defined and undefined) that participate in this process, and that understanding how these receptors work in concert will be a challenging task. Known receptors demonstrated to participate in phagocytosis of apoptotic cells by macrophages include class A scavenger receptors (124), a class B scavenger receptor, CD36, which acts in conjunction with the vitronectin receptor (125, 126), and CD14 (127) (see Figure 4).

Class A Scavenger Receptors

Scavenger receptors (SRs) are a family of structurally diverse receptors having broad ligand specificity that includes LDL, phosphatidylserine, and polyanionic

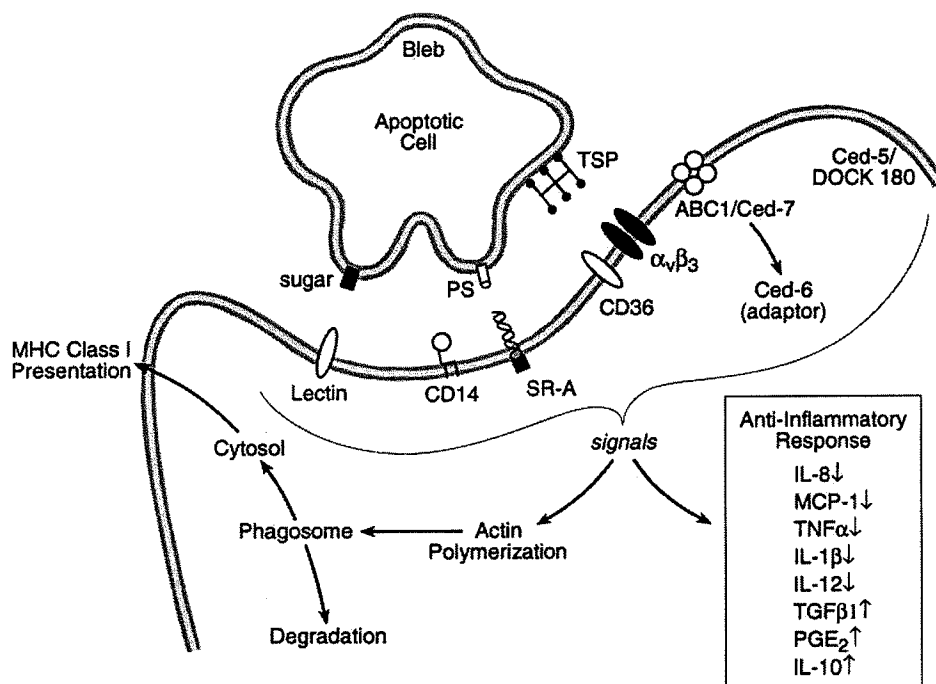


Figure 4 Phagocytosis of an apoptotic cell by a macrophage. Recognition of the apoptotic cell is mediated by a variety of receptors including lectins, CD14, scavenger receptor A (SR-A), and CD36 in conjunction with the vitronectin receptor ($\alpha_v\beta_3$). Ligands on the apoptotic cell that are recognized by these receptors include sugars, phosphatidylserine (PS), and surface-bound thrombospondin (TSP). The implicated molecules and signals are defined in the text.

compounds (128). Based on primary sequence information, scavenger receptors have been divided into at least six groups, and receptors from several of these groups are expressed on macrophages.

Scavenger receptors SR-AI and SR-AII are alternatively spliced products of the same gene. These receptors are homotrimeric glycoproteins with a short (≈ 50 amino acids) amino terminal cytoplasmic domain and a carboxy terminal extracellular ligand-binding domain, including a characteristic collagenous coiled region. SR-A binds acetylated and oxidized low-density lipoprotein, and polyanionic compounds such as maleylated bovine serum albumin and polyinosinic acid (129). Direct evidence for involvement of SR-As in macrophage phagocytosis of apoptotic cells comes from studies on the phagocytosis of apoptotic thymocytes by thymus-derived macrophages *in vitro* (124). Internalization of the thymocytes could be substantially inhibited with a monoclonal antibody to SR-A or by polyanionic ligands (124). In support of these

data, phagocytosis of thymocytes was inhibited 50% in thymic macrophages derived from SR-A null mice. Significantly, the relative number of apoptotic thymocytes in the SR-A null animals was not substantially increased, indicating that other receptors are sufficient for normal apoptotic cell clearance in the thymus (121).

Class B Scavenger Receptors and the Vitronectin Receptor

A class B scavenger receptor, CD36, has also been implicated in phagocytosis of apoptotic cells (119). CD36 is an 88-kDa membrane glycoprotein expressed on a variety of cells including platelets, monocytes, endothelial cells, and erythroblasts (130). CD36 has been identified as one of the receptors for collagen type I, thrombospondin, oxidized LDL, and phosphatidylserine (131). The role of CD36 in the internalization of apoptotic cells has best been illustrated by the observation that a monoclonal antibody to CD36 substantially inhibits the phagocytosis of aged human neutrophils by human blood-derived macrophages (132). Interestingly, murine CD36 does not mediate the uptake of apoptotic cells, and amino acids 155-183 of the human receptor were able to confer apoptotic cell binding on the mouse molecule (133). Since CD36 has a very short (4 amino acids) carboxy terminal cytoplasmic domain, it seems likely that it interacts with other transmembrane signaling molecules to stimulate phagocytosis. Thrombospondin has been suggested to bridge apoptotic cells, CD36, and the vitronectin receptor, resulting in a phagocytically active ternary complex (125, 126). Thus, the vitronectin receptor ($\alpha_v\beta_3$) may stimulate the polymerization of actin that results in CD36-dependent internalization of apoptotic cells. If this model is correct, thrombospondin is acting as an opsonin of apoptotic cells, although the mechanism by which it interacts with apoptotic cells is obscure. This cross-talk between CD36 and the vitronectin receptor illustrates the extraordinary complexity underlying phagocytosis: Here two receptors that have independent functions unrelated to phagocytosis cooperate to orchestrate a phagocytic event. Conflicting evidence exists for the importance of CD36 in phagocytizing apoptotic cells in vivo. On the one hand, blood monocytes from SLE patients demonstrate a decrease in CD36 levels paralleled by a deficiency in the phagocytosis of apoptotic cells. On the other hand, monocyte-derived macrophages from CD36-deficient patients show no defect in the phagocytosis of apoptotic neutrophils (121). This result undoubtedly reflects the considerable redundancy that underlies the uptake of apoptotic cells.

CD14

In addition to the above receptors, CD14 has also been implicated in recognition and internalization of apoptotic cells. A monoclonal antibody that specifically inhibited internalization of PMNs by monocyte-derived macrophages

was determined to recognize CD14, a molecule also known to transduce LPS signals (127). Expression of CD14 in COS cells was sufficient to mediate phagocytosis of apoptotic lymphocytes, although the uptake was relatively inefficient. Interestingly, although apoptotic cells bind to CD14 at a site close to the LPS-binding site, they do not elicit biological responses, such as TNF- α production, that are induced in macrophages by LPS (127). CD14 is a GPI-linked membrane protein and therefore must interact with other proteins to mediate signaling. Proinflammatory signaling stimulated by LPS has recently been demonstrated to be mediated by Toll-like receptor 2 (TLR2), a transmembrane receptor with homology to the IL-2 receptor (134). As for CD14, LPS signaling through TLR2 requires the serum component, LPS binding protein (LBP), and CD14 augments LPS response through TLR2 (134). This suggests that TLR2 is the transmembrane protein that associates with the LPS/LBP/CD14 ternary complex to mediate the pro-inflammatory response in macrophages. It is possible, although not yet proven, that CD14 does not interact with TLR2 during the binding of apoptotic cells, perhaps because of the absence of LBP, and that this contributes to the lack of inflammatory response during internalization.

Although macrophages express many receptors capable of recognizing determinants on apoptotic cells, it is interesting that these receptors often have functions not involving phagocytosis. In addition to redundancy, this might imply that a hierarchy of recognition mechanisms for apoptotic cells exists. Such a hierarchy might be predicated on the environment in which the events are occurring. In particular, the activation state of the macrophage might dictate the receptor system used, and this, in turn, might influence whether phagocytosis of the apoptotic cells is accompanied by the elaboration of anti-inflammatory or pro-inflammatory mediators. Thus, phagocytosis of apoptotic neutrophils by human macrophages actively inhibits LPS-induced production of a spectrum of pro-inflammatory mediators including IL-1 β , IL-8, IL-10, GM-CSF, TNF- α , leukotriene C4, and thromboxane B2 (122). The inhibition of production of inflammatory cytokines appears to be mediated by PGE₂ and TGF- β 1.

The receptor systems used to bind and internalize apoptotic cells differ depending on the activation-state of the macrophage. The uptake of apoptotic lymphocytes by activated macrophages can be inhibited by PS and N-acetylglucosamine, suggesting the involvement of lectin-like receptors (135), while the integrin-binding peptide, RGDS, and cationic amino acids and sugars have little blocking activity. The opposite appears true for unactivated macrophages; phagocytosis of apoptotic lymphocytes by unactivated macrophages can be blocked by RGDS and cationic amino acids and sugars, while PS and N-acetylglucosamine have little effect (135). This has been proposed to suggest a role for the vitronectin receptor in phagocytosis of apoptotic cells by unactivated macrophages but not by activated macrophages. In both cases,

an antibody to CD14 inhibits uptake (127). Thus, activated and nonactivated macrophages phagocytize the same apoptotic cells via different mechanisms. In addition, the same macrophage will internalize different apoptotic cells by different mechanisms. This is supported by the observation that ligation of CD44 augments apoptotic neutrophil uptake but not uptake of apoptotic lymphocytes (136). Conversely, the uptake of apoptotic lymphocytes by peritoneal macrophages can be blocked by phosphatidyl serine, while the uptake of apoptotic neutrophils is unaffected by this phospholipid (137).

C. elegans as a Genetic System

The complex interactions and downstream signals resulting in the uptake of apoptotic cells may best be deciphered using genetic systems applicable to phagocytosis. A variety of *C. elegans* mutants defective in apoptotic cell clearance have been described, and while this phagocytosis is performed by neighboring cells in the worm, much of what has been learned will likely be useful in dissecting out the pathway in macrophages. Mutations in *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10* result in a large increase in persistent apoptotic bodies in *C. elegans*. Genetic analysis suggests that the six engulfment genes fall into two groups: *ced-1*, *ced-6*, and *ced-7* are in one group, while *ced-2*, *ced-5*, and *ced-10* are in the other (138). Single and double mutants within the same group show relatively weak defects, while double mutants between the two groups show severe phagocytic defects (138). Thus, the two groups of genes might be involved in two distinct, but partially redundant pathways in phagocytosis.

Ced-5 encodes a protein with homology to the mammalian protein DOCK180 and the *Drosophila* protein myoblast city (MBC) (139). These proteins, collectively known as the CDM family, appear to regulate cytoskeletal-membrane interactions and, in particular, membrane extension. DOCK180 interacts with the adaptor protein *crk* and has been implicated in integrin-mediated signaling and cell movement (140). Expression of human DOCK180 in *C. elegans* rescues the cell migration defects, but does not restore the uptake of apoptotic cells, suggesting that the proteins are at least partially interchangeable (139). *Drosophila* MBC is necessary for myoblast fusion and for the migration of a population of epithelial cells (141).

Ced-7 has protein sequence homology to ABC transporters, proteins that translocate a wide variety of substrates across membranes (142). Interestingly, ABC1, a member of the ABC transporters, has been identified in macrophages, and the ability of macrophages to ingest apoptotic thymocytes, but not yeast cells, is impaired when the macrophages are treated with antibodies to ABC1 (143). *Ced-7* acts in both the target cell and the engulfing cell, and it has been suggested to be important for the interaction between the cells (142).

Overexpression of Ced-6 can partially restore phagocytosis in ced-1 or ced-7 deficient cells, suggesting that it acts downstream of these two genes (144). Ced-6 appears to be an adaptor molecule since it contains a phosphotyrosine-binding (PTB) domain at its N-terminus and a proline-rich domain capable of interacting with an SH3 domain at its C-terminus (144).

Implications for Immunity

Bhardwaj and coworkers recently reported that dendritic cells, but not macrophages, are capable of stimulating class I-restricted CD8+ cytotoxic T lymphocytes, by efficiently presenting antigen derived from phagocytized apoptotic cells (145). This pathway might account for the *in vivo* phenomenon of cross-priming, whereby antigens derived from tumor cells or transplants are presented by host antigen presenting cells (146, 147). In addition, this pathway could facilitate the presentation of self-antigens, resulting in the breaking of tolerance and the activation of autoimmune disease (148). The very different outcomes of ingestion of apoptotic cells by macrophages or dendritic cells once again highlight the heterogeneous nature of phagocytosis and suggest that understanding the differences in phagocytic mechanisms between these cell types could lead to new therapeutic strategies for a number of inflammatory diseases.

CONCLUSIONS

It is clear that phagocytosis in macrophages is a diverse process; the signals leading to actin polymerization and particle internalization depend on the specific receptors that mediate the process and on additional modifying signals that can be generated by complex particles. Complex particles, such as bacteria, can activate multiple receptors whose signaling pathways may interact in intricate and unpredictable ways. In addition, living bacteria have the capacity to modify signaling pathways within eukaryotic cells. For example, *S. typhimurium* can introduce a GDP:GTP exchange factor into cells that modifies the way that Rho family of GTPases signals the actin cytoskeleton (93), while *Yersinia* species introduce a broad spectrum tyrosine phosphatase (90).

In addition to the complexity relating to the different receptor systems and the capacity of microbes to modify phagocytosis, it is important to recognize that the formation of the phagosome can be a heterogeneous process, even when a single cell ingests two identical particles. While all phagocytosis involves actin remodeling around the phagocytic cup, we have observed that certain cytoskeletal proteins that decorate a particular phagosome are absent from otherwise identical phagosomes in the same cell. These differences are not temporal but are likely to arise from stochastic, or even chaotic, processes. For example, actin cross-linking can be achieved using any of the diverse number

of actin cross-linking proteins that are expressed in the cell. Thus, if a specific actin cross-linking protein happens to have been enriched in a specific region of the cell, for example the leading edge of a motile cell, it may be used during phagocytosis at the leading edge while another protein may serve the same function on a phagosome formed at the trailing edge. It is clear that the molecular dissection of phagocytosis represents a daunting task; more than 100 actin-binding proteins have been identified, and many of these are expressed in the same cell. Two common approaches that have been used to establish the role of specific gene products in a particular biological phenomenon have been to express dominant negative forms of the protein, or to delete the gene encoding the protein. However, the interpretation of the results is complicated by the observation that these two approaches do not always yield the same phenotype. Genetically tractable organisms that allow the dissection of phagocytosis will contribute greatly to our ability to make sense of this complexity. *C. elegans* has already extended our understanding of the phagocytosis of apoptotic cells, even though this organism does not have macrophages. *Dictyostelium discoideum* is a free living, haploid organism that phagocytizes bacteria and that has been used to great effect in dissecting cell motility. Gerish and coworkers have recently turned their attention to phagocytosis in *D. discoideum*, and they have almost immediately elucidated new pathways and molecules that are reiterated in mammalian systems (149–151).

Ultimately, phagocytosis, like most other problems in biology, will have to be analyzed as a complex system rather than a linear series of isolated enzymatic reactions. In this guise, phagocytosis provides a window into the coordinate functioning of the actin and tubulin based cytoskeletons, and it could serve as a model system for analyzing diverse biological phenomena including synaptic transmission, mitogenesis, and morphogenesis.

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CONTENTS

| | |
|--|-----|
| Discovering the Origins of Immunological Competence, <i>Jacques F. A. P. Miller</i> | 1 |
| Multifaceted Regulation of IL-15 Expression and Its Role in NK Cell Differentiation & Host Response to Intracellular Pathogens, <i>T. A. Waldmann, Y. Tagaya</i> | 19 |
| Immunodominance in Major Histocompatibility Complex Class I-Restricted T Lymphocyte Responses, <i>Jonathan W. Yewdell, Jack R. Bennink</i> | 51 |
| Integration of TCR-Dependent Signaling Pathways by Adapter Proteins, <i>James L. Clements, Nancy J. Boerth, Jong Ran Lee, Gary A. Koretzky</i> | 89 |
| Evolution of Antigen Binding Receptors, <i>Gary W. Litman, Michele K. Anderson, Jonathan P. Rast</i> | 109 |
| Transcriptional Regulation of T Lymphocyte Development and Function, <i>Chay T. Kuo, Jeffrey M. Leiden</i> | 149 |
| Natural Killer Cells in Antiviral Defense: Function and Regulation by Innate Cytokines, <i>Christine A. Biron, Khuong B. Nguyen, Gary C. Pien, Leslie P. Cousens, Thais P. Salazar-Mather</i> | 189 |
| Mature T Lymphocyte Apoptosis--Immune Regulation in a Dynamic and Unpredictable Antigenic Environment, <i>Michael Lenardo, Francis Ka-Ming Chan, Felicita Hornung, Hugh McFarland, Richard Siegel, Jin Wang, Lixin Zheng</i> | 221 |
| Immunologic Basis of Antigen-Induced Airway Hyperresponsiveness, <i>Marsha Wills-Karp</i> | 255 |
| Regulation of T Cell Fate by Notch, <i>Ellen Robey</i> | 283 |
| The CD1 System: Antigen Presenting Molecules for T Cell Recognition of Lipids and Glycolipids, <i>Steven A. Porcelli, Robert L. Modlin</i> | 297 |
| Tumor Necrosis Factor Receptor and Fas Signaling Mechanisms, <i>D. Wallach, E. E. Varfolomeev, N. L. Malinin, Yuri V. Goltsev, A. V. Kovalenko, M. P. Boldin</i> | 331 |
| Structural Basis of T Cell Recognition, <i>K. Christopher Garcia, Luc Teyton, Ian A. Wilson</i> | 369 |
| Development and Maturation of Secondary Lymphoid Tissues, <i>Yang-Xin Fu, David D. Chaplin</i> | 399 |
| The Structural Basis of T Cell Activation by Superantigens, <i>Hongmin Li, Andrea Llera, Emilio L. Malchiodi, Roy A. Mariuzza</i> | 435 |
| The Dynamics of T Cell Receptor Signaling: Complex Orchestration and the Key Roles of Tempo and Cooperation, <i>Ronald N. Germain, Irena Stefanová</i> | 467 |
| The Regulation of CD4 and CD8 Coreceptor Gene Expression During T Cell Development, <i>Wilfried Ellmeier, Shinichiro Sawada, Dan R. Littman</i> | 523 |
| Genetic Analysis of B Cell Antigen Receptor Signaling, <i>Tomohiro Kurosaki</i> | 555 |
| Mechanisms of Phagocytosis in Macrophages, <i>Alan Aderem, David M. Underhill</i> | 593 |
| Population Biology of HIV-1 Infection: Viral and CD4+ T Cell Demographics and Dynamics in Lymphatic Tissues, <i>A. T. Haase</i> | 625 |

| | |
|---|-----|
| Chemokine Receptors as HIV-1 Coreceptors: Roles in Viral Entry, Tropism, and Disease, <i>Edward A. Berger, Philip M. Murphy, Joshua M. Farber</i> | 657 |
| The IL-4 Receptor: Signaling Mechanisms and Biologic Functions, <i>Keats Nelms, Achsa D. Keegan, José Zamorano, John J. Ryan, William E. Paul</i> | 701 |
| Degradation of Cell Proteins and the Generation of MHC Class I-Presented Peptides, <i>Kenneth L. Rock, Alfred L. Goldberg</i> | 739 |
| The Central Effectors of Cell Death in the Immune System, <i>Jeffrey C. Rathmell, Craig B. Thompson</i> | 781 |
| Selection of the T Cell Repertoire, <i>Eric Sebzda, Sanjeev Mariathasan, Toshiaki Ohteki, Russell Jones, Martin F. Bachmann, Pamela S. Ohashi</i> | 829 |
| Regulation of Immune Responses Through Inhibitory Receptors, <i>Eric O. Long</i> | 875 |
| The Wiskott-Aldrich Syndrome Protein (WASP): Roles in Signaling and Cytoskeletal Organization, <i>Scott B. Snapper, Fred S. Rosen</i> | 905 |
| The High Affinity IgE Receptor (Fc Epsilon RI): From Physiology to Pathology, <i>Jean-Pierre Kinet</i> | 931 |
| THE CRYSTAL STRUCTURE OF THE HUMAN HIGH-AFFINITY IgE RECEPTOR (Fc epsilon RI alpha), <i>Scott C. Garman, Jean-Pierre Kinet, Theodore S. Jardetzky</i> | 973 |